APPENDIX A

Treatment of Animal Model of Experimental Autoimmune Encephalomyelitis using β-glucuronidase and myelin increases remission

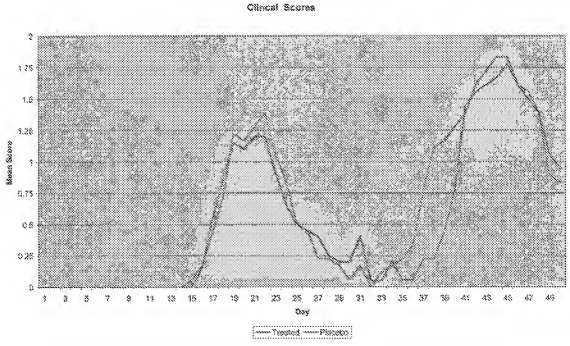
Animals (mice 5-8 weeks of age) were challenged to induce Experimental Autoimmune Encephalomyelitis (EAE), an animal model of multiple sclerosis. All animals were monitored for disease during the acute disease phase and any animal not showing signs of disease was excluded from further study. Treatments (Group A- chondroitin-6-sulphate in buffered saline, Placebo; Group B- β-glucuronidase, myelin peptides, and chondroitin-6-sulphate in buffered saline) were given during the first remission phase by a single subcutaneous injection to the neck. After treatment, all animals were randomised into cages.

Incidence of clinical disease symptoms

The incidence of disease symptoms during the acute phase among all animals was 90%, with the peak incidence of disease occurring on day 21 post challenge. One animal in the treatment group failed to show any signs of disease throughout the course of the experiment. A total of 72.5% of the animals went on to show some signs of recurrent disease with a peak of 57.5% on day 46. Thus, approximately 83% of those animals that developed acute disease symptoms went on to relapse. The animal which did not develop acute disease was excluded from further data analysis. Of the remaining animals approximately 70% developed recurrent disease.

Phenytoin protects spinal cord axons and preserves axonal conduction and neurological function in a model of neuroinflammation in vivo. (*J Neurophysiol* 90:3566-71.) on a 0-6 scale, with 0.5 gradations for intermediate scores, as follows: 0 = normal without clinical signs; 1 = flaccid tail; 2 = abnormal righting reflex; 3 = partial hindlimb paralysis; 4 = complete hindlimb paralysis; 5 = moribund; and 6 = death. The animals were treated when all animals entered the remission phase. The remission phase was deemed to have started when the average clinical score across all animals was less than 0.75, and hence fell on day 25.

The results of the scoring are shown in Figure 1. As can be seen in the data, the animals in the treated group remained in remission (were scored below 0.75) until day 40 while the animals in the placebo group remained in remission until day 37. Hence, the animals in the placebo group spent 12 days in remission and the animals in the treated group spent 15 days in remission, an extra three days which represents a 25% improvement over placebo at a single dose.



METHODOLOGY

Groups of 10 Biozzi mice aged between 5 and 8 weeks of age had transponders fitted as an aid to identification. Animals were challenged firstly in the flank (day 0) with whole spinal cord homogenate in Complete Freund's Adjuvant (CFA) and secondly in the shoulder area (day 7) in order to induce Experimental Autoimmune Encephalomyelitis (EAE). All animals were monitored for disease during the acute disease phase and any animal not showing signs of disease was excluded from further study. Treatments were given during the first remission phase by subcutaneous injection on a single occasion to the scruff of the neck.

The remission phase was defined starting when the average clinical score across all animals was less than 0.75. After treatment, all animals were randomized into cages. Scoring of

EAE was performed daily as per standard scoring system using transponder identification to determine animal number. The individual performing the scoring was aware only of the animal number as determined from the transponder, and scores were collated by a separate member of the laboratory staff in order to ensure complete blinding of the study. Moribund animals were euthanized according to Home Office regulations. Any remaining healthy animals were euthanized at day 50 post-induction. Spinal cords and brains were fixed in neutral buffered formalin for histopathology.

Experimental groups *(n=10/group)*

A: Placebo treated control

B: Treatment group

Scoring of EAE was performed daily as per standard scoring system blinding of the study.

Treatments

A- Enzyme 0.3 ml placebo vials of buffered saline (pH 5.9)

B- Enzyme 0.3 ml vials of activated β-glucuronidase in buffered saline (pH 5.9)

A- Antigen 1 ml placebo vials of 0.5 mg/ml chondroitin-6-sulphate in buffered saline (pH 5.9)

B- Antigen 1 ml vials containing

0.113 µg/m1 peptide YLATASTMDHARHGFLPRHRDTGI,

0.069 µg/ml peptide PGYPIRALVGDEQED,

0.073 µg/ml peptide YLINVIHAFQYVIGT

in 0.5 mg/ml chondroitin-6-sulphate in buffered saline (pH5.9) ('C' strength)

The vials were clearly labelled as Enzyme for groups A or B and Antigens for groups A or B but the researchers were blinded to which group corresponded to which treatment. The treatments were stored at 4°C until use and kept on ice following removal from refrigerator and up to the point of injection. Treatments were thoroughly mixed at a ratio of 0.2m1 enzyme and 0.8ml antigen peptides in a lml syringe no longer than 10 minutes prior to injection of 100p1 into each animal.

Clinical disease severity

Both groups of mice gave a similar average disease scores during the acute disease phase, prior to treatment. Immunized mice were observed daily for clinical signs and scored as described by Lo et *al.* (2003) (Lo AC, Saab CY, Black JA, Waxman SG. (2003)

Endpoints

Clinical score of paralysis: Animals were scored for signs of EAE (scale 0 to 5) daily from day 10 following initial challenge spinal cord homogenate in CFA. Data are provided graphed to show incidence and severity for each group over time.

Statistical analysis

The two endpoints were analysed as follows:

Clinical score of paralysis

Clinical paralysis scores were recorded for each animal using a 0 to 5 scale, and this was performed daily from Day 10 by at most two scorers. Prior to analysis, data for each animal was aggregated as follows:

An animal average paralysis score was calculated for a baseline period and an end of study period, each lasting three days. i.e.

(i) Average of days n, n+1 and n+2 where n is the day where the average clinical paralysis scores were less than 0.75.

(ii) Average of days 48, 49 and 50

This was performed separately for each scorer.